



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) EP 0 343 950 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
18.10.2000 Bulletin 2000/42

(51) Int. Cl.⁷: C07K 14/00, C12N 15/13,
G01N 33/53

(21) Application number: 89305241.5

(22) Date of filing: 24.05.1989

(54) Human receptor Fc(gamma)RIII
Humaner Rezeptor Fc(gamma)RIII
Récepteur humain Fc(gamma)RIII

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(30) Priority: 27.05.1988 US 199513
19.01.1989 US 300039

(43) Date of publication of application:
29.11.1989 Bulletin 1989/48

(60) Divisional application:
97106398.7 / 0 791 653

(73) Proprietor:
Applied Research Systems ARS Holding N.V.
Curacao (AN)

(72) Inventors:
• Peltz, Gary A.
San Francisco, CA 94115 (US)
• Moore, Kevin W.
Palo Alto, CA 94306 (US)

(74) Representative:
Schlich, George William et al
Mathys & Squire
European Patent Attorneys,
100 Gray's Inn Road
London WC1X 8AL (GB)

(56) References cited:
WO-A-87/07277

- THE NEW ENGLAND JOURNAL OF MEDICINE,
vol. 314, no. 19, 08 May 1986,
Massachusetts Medical Society; S. B.
CLARKSON et al., pp. 1236-1239
- NATURE, vol. 333, 9th June 1988, D. SIMMONS et
al., pp. 568-570

Remarks:

Consolidated with 89906578.3/0449828 (European
application No./publication No.) by decision dated
20.10.93.

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 0 343 950 B1

BEST AVAILABLE COPY

ATTORNEY DOCKET NUMBER: 11183-003-999
SERIAL NUMBER: 10/524,134
REFERENCE: B09

Description

Field of the Invention

[0001] The invention relates generally to therapeutic compounds, and more particularly to soluble and membrane-bound forms of a low-affinity receptor for human immunoglobulin G, nucleic acids encoding the same, and diagnostic and therapeutic uses of such receptors.

BACKGROUND

[0002] Receptors for the Fc portion of immunoglobulin G (IgG) play a central role in cellular immune defenses. Three types of such receptors have been identified: A 72 kilodalton (kD) receptor with high affinity for monomeric IgG is found on monocytes and some macrophages, a 40 kD receptor with low affinity for monomeric IgG is found on monocytes, neutrophils, eosinophils, platelets and certain human tumor-cell lines, and a 50-70 kD receptor with low affinity for monomeric IgG is found on neutrophils, eosinophils, natural killer cells, and macrophages. These three types of Fc γ receptor are referred to as Fc γ RI, Fc γ RII, and Fc γ RIII, respectively: Unkeless et al., Ann. Rev. Immunol., Vol. 6, pgs. 251-281 (1988).

[0003] It is believed that Fc γ RIII-mediated removal of IgG-coated platelets plays an important part in the pathogenesis of immune thrombocytopenic purpura (ITP), a platelet-deficiency condition characterized by excessive bleeding: von dem Borne, pgs. 222-256, in Immunohaematology, Engelfriet et al., eds. (Elsevier, Amsterdam, 1984). Clarkson et al., New England J. Med., Vol. 314, pgs. 1236-1239 (1986), report that the infusion of ligand-blocking anti-Fc γ RIII antibody into a patient with refractory ITP resulted in a transient increase in platelet count. This observation suggests that the most deleterious manifestation of ITP could be temporarily ameliorated by the administration of agents that block or compete with Fc γ RIII for binding sites on IgG-coated platelets.

[0004] In a separate area of clinical immunology, elevated serum levels of aggregates consisting of immunoglobulin and antigen (so-called "immune complexes") have been correlated with a wide variety of disorders, particularly autoimmune diseases, such as systemic lupus erythematosus (SLE), and rheumatoid arthritis. The level of such complexes has become an important diagnostic for presence of autoimmune disorders; e.g. Theofilopoulos et al., Am. J. Pathol., Col. 100, pgs. 531-591 (1980).

[0005] Present assays for the serum level of immune complexes include solid-phase assays which take advantage of the affinity of the complexes for certain complement or rheumatoid factor proteins, and cellular assays which take advantage of the property of Raji cells to preferentially bind immune complexes; The-

ofilopoulos et al., chapter 28, and Toth et al., chapter 29, in Rose et al., eds. Manual of Clinical Immunology, 3rd Ed. (American Society for Microbiology, Washington, D.C., 1986). Unfortunately, like many mammalian-cell based assays, the Raji-cell assay is difficult to perform, and requires elaborate controls and standards because of the inherent variability of Raji-cell binding to immune complexes.

[0006] In the light of the foregoing, it would be advantageous for the medical community to have alternative assay methods for immune complexes utilizing well characterized, widely available, and conveniently cultured cell lines. It would also be advantageous if soluble Fc γ Rs could be produced in sufficient quantity to permit practical emergency therapy for refractory cases of ITP.

[0007] The present invention is directed to an isolated nucleic acid having a sequence of nucleotides which encodes soluble or membrane-bound human Fc γ RIII, to the nucleic acid contained within pcD(SR α)-GP5, to the use of these nucleic acids in production of Fc γ RIII and to methods of Fc γ RIII production.

[0008] The invention also relates to diagnostic and therapeutic uses of such polypeptides.

[0009] The invention also relates to diagnostic and therapeutic uses of such polypeptides.

[0010] The invention is based on the discovery of a cDNA encoding a human Fc γ RIII. A cDNA clone, pcD(SR α) containing the Fc γ RIII-encoding insert (illustrated in Figure 1) is deposited in E. coli K12 strain MC1061 with the American Type Culture Collection (ATCC) Rockville, Maryland, USA, under accession number 67707.

[0011] The nucleotide and amino acid sequence of a cDNA encoding an Fc γ RIII is disclosed in Simmons et al. in Nature, Vol. 333 dated 9 June 1988, based on sequence analysis of a plasmid designated pCD16.

[0012] Sequence analysis of our pcD(SR α) provides the following amino acid sequences for soluble or membrane-bound Fc γ RIII: Arg - Thr - Glu - Asp - Leu - Pro - Lys - Ala - Val - Val - Phe - Leu - Glu - Pro - Gln - Trp - Tyr - Arg - Val - Leu - Glu - Lys - Asp - Ser - Val - Thr - Leu - Lys - Cys - Gln - Gly - Ala - Tyr - Ser - Pro - Glu - Asp - Asn - Ser - Thr - Gln - Trp - Phe - His - Asn - Glu - Asn - Leu - Ile - Ser - Ser - Gln - Ala - Ser - Ser - Tyr - Phe - Ile - Asp - Ala - Ala - Thr - Val - Asp - Asp - Ser - Gly - Glu - Tyr - Arg - Cys - Gln - Thr - Asn - Leu - Ser - Thr - Leu - Ser - Asp - Pro - Val - Gln - Leu - Glu - Val - His - Val - Gly - Trp - Leu - Leu - Leu - Gln - Ala - Pro - Arg - Trp - Val - Phe - Lys - Glu - Glu - Asp - Pro - Ile - His - Leu - Arg - Cys - His - Ser - Trp - Lys - Asn - Thr - Ala - Leu - His - Lys - Val - Thr - Tyr - Leu - Gln - Asn - Gly - Lys - Asp - Arg - Lys - Tyr - Phe - His - His - Asn - Ser - Asp - Phe - His - Ile - Pro - Lys - Ala - Thr - Leu - Lys - Asp - Ser - Gly - Ser - Tyr - Phe - Cys - Arg - Gly - Leu - Val - Gly - Ser - Lys - Asn - Val - Ser - Ser - Glu - Thr - Val - Asn - Ile - Thr - Ile - Thr - Gln - Gly - Leu - Ala - Val - Ser - Thr - Ile - Ser - Ser - Phe - Ser - Pro -

Pro - Gly

Formula I

or

Arg - Thr - Glu - Asp - Leu - Pro - Lys - Ala - Val - Val -
Phe - Leu - Glu - Pro - Gln - Trp - Tyr - Arg - Val - Leu -
Glu - Lys - Asp - Ser - Val - Thr - Leu - Lys - Cys - Gln -
Gly - Ala - Tyr - Ser - Pro - Glu - Asp - Asn - Ser - Thr -
Gln - Trp - Phe - His - Asn - Glu - Ser - Leu - Ile - Ser -
Ser - Gln - Ala - Ser - Ser - Tyr - Phe - Ile - Asp - Ala -
Ala - Thr - Val - Asp - Asp - Ser - Gly - Glu - Tyr - Arg -
Cys - Gln - Thr - Asn - Leu - Ser - Thr - Leu - Ser - Asp -
Pro - Val - Gln - Leu - Glu - Val - His - Ile - Gly - Trp -
Leu - Leu - Leu - Gln - Ala - Pro - Arg - Trp - Val - Phe -
Lys - Glu - Glu - Asp - Pro - Ile - His - Leu - Arg - Cys -
His - Ser - Trp - Lys - Asn - Thr - Ala - Leu - His - Lys -
Val - Thr - Tyr - Leu - Gln - Asn - Gly - Lys - Gly - Arg -
Lys - Tyr - Phe - His - His - Asn - Ser - Asp - Phe - Tyr -
Ile - Pro - Lys - Ala - Thr - Leu - Lys - Asp - Ser - Gly -
Ser - Tyr - Phe - Cys - Arg - Gly - Leu - Phe - Gly - Ser -
Lys - Asn - Val - Ser - Ser - Glu - Thr - Val - Asn - Ile -
Thr - Ile - Thr - Gln - Gly - Leu - Ala - Val - Ser - Thr - Ile -
Ser - Ser - Phe - Phe - Pro - Pro - Gly;

Formula II

together with variant proteins wherein 0 or 1 amino acid
residue is deleted, and 0-2 amino acid residues, other
than Cys, Phe, Trp and Tyr, are replaced according to
the following substitution Table:

Amino Acid	Can be replaced by
Ala	Ser, Thr or Gly
Arg	Lys
Asn	Asp or Ser
Asp	Glu or Asn
Gln	Glu
Glu	Asp or Gln
Gly	Ala or Ser
His	Asn
Ile	Val or Leu
Leu	Val or Ile
Lys	Arg or Asn
Met	Leu
Pro	Ala
Ser	Ala, Thr, Gly or Asn
Thr	Ser, Ala or Lys
Val	Ile, Ala or Leu.

[0013] For the variant proteins, this substitution
Table presents the group of additional L-amino acids

that are synonymous to the amino acids that can be
replaced. Synonymous amino acids within a group have
sufficiently similar physiochemical properties for substi-
tution between members of the group to preserve the
biological function of the molecule, Grantham, Science,
Vol. 185, pgs. 862-864 (1974); and Dayhoff et al., Atlas
of Protein Sequence and Structure 1972, Vol. 5, pgs.
89-99.

[0014] Furthermore, it is clear that deletions of
amino acids may also be made in the above-identified
sequences without altering biological function, particu-
larly if only one amino acid is deleted and amino acids
that are critical to a functional conformation are not
removed or displaced, e.g. some cysteine residues:
Anfisen, "Principles That Govern The Folding of Protein
Chains", Science, Vol. 181, pgs. 223-230 (1973). Pro-
teins and muteins produced by deletions are within the
purview of the present invention. Whenever an amino
acid residue of a protein of Formula I or II is referred to
herein by number, such a number is in reference to the
N-terminus of the protein.

[0015] In particular, 1-2 amino acid residues (espe-
cially 1) other than Arg, Gln, His, Met and Pro, can be
replaced according to the following substitution Table:

Amino Acid	Can be replaced by
Ala	Ser
Asn	Asp
Asp	Glu
Glu	Asp
Gly	Ala
Ile	Val
Leu	Val
Lys	Arg
Ser	Ala or Thr
Thr	Ser or Ala
Val	Ile.

[0016] As used herein, "N-fold substituted" in refer-
ence to Formula I or II describes a group of amino acid
sequences differing from the native amino acid
sequences thereof by zero-N substitutions, such that
the replacing amino acid is selected from the appropri-
ate group of amino acids synonymous to the amino acid
at the location of the substitution. That is, if the amino
acid residue Ala is at the location for substitution, then
that Ala can be replaced with Ser, Thr, or Gly.

[0017] Likewise, as used herein, the term "N-fold
deleted" in reference to Formula I or II describes a
group of amino acid sequences differing from the native

amino acid sequences thereof by zero-N deletions of amino acids.

[0018] Throughout, standard abbreviations are used to designate amino acids, nucleotides, restriction endonucleases, and the like; e.g. Cohn, "Nomenclature and Symbolism of α -Amino Acids," Methods in Enzymology, Vol. 106, pgs. 3-17 (1984); Wood et al. Biochemistry: A Problems Approach, 2nd ed. (Benjamin, Menlo Park, 1981); and Roberts, "Directory of Restriction Endonucleases", Methods in Enzymology, Vol. 68, pgs. 27-40 (1979).

[0019] A particularly preferred feature of the present invention is that part of the protein that consists of the extracellular domain of a human Fc γ RIII.

Brief Description of the Drawings

[0020]

Figure 1, parts A and B (on two sheets to be read side-by-side) illustrates the nucleotide sequence of the cDNA insert of pcD(SR α)-GP5;

Figure 2 illustrates the relative location of the stop codon inserted to produce a soluble human Fc γ RIII, secreted as an Fc γ RIII mutant;

Figure 3 parts A and B (on two sheets to be read side-by-side) illustrates the nucleotide sequence of the cDNA insert of pcD(SR α)-NL10.

DETAILED DESCRIPTION OF THE INVENTION

[0021] The present invention includes nucleic acids encoding polypeptides capable of binding of the Fc portion of human IgG. These polypeptides are derived from human Fc γ RIII. The invention includes soluble and membrane-bound polypeptides for human Fc γ RIII. These and other modified versions of the polypeptides are readily produced using standard protein engineering techniques.

[0022] Once nucleic acid sequence and/or amino acid sequence information is available for a native protein, a variety of techniques become available for producing virtually any mutation in the native sequence. Shortle, in Science, Vol. 229, pgs. 1193-1201 (1985), reviews techniques for mutating nucleic acids which are applicable to the present invention. Preferably, mutants of the proteins of the present invention are produced by site-specific oligonucleotide-directed mutagenesis, e.g. Zoller and Smith, Methods in Enzymology, Vol. 100, pgs. 468-500 (1983), and Mark et al., U.S. Patent 4,518,584 entitled "Human Recombinant Interleukin-2 Muteins", or by so-called "cassette" mutagenesis described by Wells et al. in Gene, Vol. 34, pgs. 315-323 (1985), by Estell et al. in Science, Vol. 233, pgs. 659-663 (1986), and also essentially by Mullenbach et al. in J. Biol. Chem., Vol. 261, pgs. 719-722 (1986), and by Feretti et al. in Proc. Natl. Acad. Sci., Vol. 83, pgs. 597-603 (1986).

[0023] Polypeptides with amino acid modifications (i.e. muteins) may be desirable in a variety of circumstances. For example, undesirable side effects might be reduced by certain muteins, particularly if the side effect is associated with a different part of the polypeptide from that of the desired activity. In some expression systems, the native polypeptide may be susceptible to degradation by proteases. In such cases, selected substitutions and/or deletions of amino acids which change the susceptible sequences can significantly enhance yields, e.g. British patent application 2173-804-A where Arg at position 275 of human tissue plasminogen activator is replaced by Gly or Glu. Muteins may also increase yields in purification procedures and/or increase shelf lives of proteins by eliminating amino acids susceptible to oxidation, acylation, alkylation, or other chemical modifications. For example, methionine readily undergoes oxidation to form a sulfoxide, which in many proteins is associated with loss of biological activity; e.g. Brot and Weissbach, Arch. Biochem. Biophys., Vol. 223, pg. 271 (1983). Methionines can often be replaced by more inert amino acids with little or no loss of biological activity, e.g. Australian patent application AU-A-52451/86. In bacterial expression systems, yields can sometimes be increased by eliminating or replacing conformationally inessential cysteine residues, e.g. Mark et al., U.S. Patent 4,518,584.

[0024] Preferably, soluble forms of the Fc γ RIII of the invention are produced by introducing a stop codon prior to (i.e. in the 5'- or "upstream" direction of) the coding region for the transmembrane and intracellular portions of the Fc γ RIII cDNA. This is conveniently done by site-specific mutagenesis. Transmembrane regions are readily identified by the presence of an amino acid segment containing from about 20-25 residues having high average hydrophobicity, e.g. Wickner, Science, Vol. 210, pgs. 861-868 (1980), and Greene et al., Ann. Rev. Immunol., Vol. 4, pgs. 69-95 (1986).

[0025] Plasmid pcD(SR α)-GP5 is similar to the pcD shuttle vector described by Okayama and Berg, Mol. Cell. Biol., Vol. 2, pgs. 161-170 (1983), and Vol. 3; pgs. 280-289 (1983), except that the SV40 promoter has been modified to improve expression by the downstream insertion of a portion of the long terminal repeat (LTR) from a HTLV(I) retrovirus, described by Takebe et al., Mol. Cell. Biol., Vol. 8, pgs. 466-472 (1988). The plasmid is conveniently propagated in E. coli K12 strain MC1061, or like host.

[0026] The immunoglobulin G binding property of the Fc γ RIII of the invention is measured by standard techniques, e.g. (1) in the case of membrane-bound Fc γ RIII: the ability of cells transfected with the Fc γ RIII cDNA to form rosettes in the presence of IgG-coated red blood cells (RBCs) or to preferentially bind human IgG aggregated by heat treatment; or (2) in the case of soluble Fc γ RIII: the ability to preferentially remove Fc γ RIII from solution by an immunoabsorbent column comprising human IgG, or to inhibit rosette formation

between IgG-coated RBCs and cells known to have FcγRIII. The former measurements can be made with fluorescently labeled human IgG molecules, e.g. Haugland, Handbook of Fluorescent Probes (Molecular Probes, Inc., Junction City, OR, 1985). The latter measurements can be made by constructing an IgG column from isolated human IgG and a commercially available activated sepharose column, e.g. from Bio-Rad Laboratories (Richmond, CA).

[0027] Rosette assays are standard in the art; e.g. Winchester et al., chapter 31, in Rose et al., eds., Manual of Clinical Laboratory Immunology, 3rd Ed. (American Society for Microbiology, Washington, D.C., 1986).

[0028] Once the cDNA of the invention has been cloned, a wide range of expression systems (i.e. combinations of host and expression vector) can be used to produce the proteins of the invention. Possible types of host cells include, but are not limited to, bacterial, yeast, insect, mammalian, and the like. Selecting an expression system, and optimizing protein production thereby, involves the consideration and balancing of many factors, including (1) the nature of the protein to be expressed, e.g. the protein may be poisonous to some host organisms, it may be susceptible to degradation by host proteases, or it may be expressed in inactive conformations or in insoluble form in some hosts, (2) the nature of the messenger RNA (mRNA) corresponding to the protein of interest, e.g. the mRNA may have sequences particularly susceptible to host endonucleases, which drastically reduce the functional lifetime of the mRNA, or the mRNA may form secondary structures that mask the start codon or ribosome binding site, thereby inhibiting initiation of translation in some hosts, (3) the selection, availability, and arrangement of host-compatible expression-control sequences in the 3'- and 5'-regions flanking the coding region -- these include promoters, 5'- and 3'-protector sequences, ribosome binding sites, transcription terminators, enhancers, polyadenylate addition sites, cap sites, intron-splice sites, and the like, (4) whether the protein has a secretion-signal sequence which can be processed by the host, or whether an expression-control sequence encoding a signal sequence endogenous to the host must be spliced onto the region encoding the mature protein, (5) the available modes and efficiencies of transfection or transformation of the host, and whether transient or stable expression is desired, (6) the scale and cost of the host culture system desired for expressing the protein, (7) whether, and what type of, posttranslational modifications are desired; e.g. the extent and kind of glycosylation desired may affect the choice of host, (8) the ease with which the expressed protein can be separated from proteins and other materials of the host cells and/or culture medium e.g. in some cases it may be desirable to express a fusion protein with a specialized signal sequence to aid in later purification steps, e.g. Sassenfeld et al., Biotechnology, January 1984, (9) the stability and copy number of a

particular vector in a selected host, e.g. Hofschneider et al., eds. Gene Cloning in Organisms Other than E. coli (Springer Verlag, Berlin, 1982) and (10) like factors known to those skilled in the art.

[0029] Many reviews are available which provide guidance for making choices and/or modifications of specific expression systems in light of the recited factors, e.g., de Boer and Shepard, "Strategies for Optimizing Foreign Gene Expression in Escherichia coli" pgs. 205-247, in Kroon, ed. Genes: Structure and Expression (John Wiley & Sons, New York, 1983), review several E. coli expression systems; Kucherlapati et al., Critical Reviews in Biochemistry, Vol. 16, Issue 4, pgs. 349-379 (1984), and Banerji et al., Genetic Engineering, Vol. 5, pgs. 19-31 (1983) review methods for transfecting and transforming mammalian cells; Reznikoff and Gold, eds., Maximizing Gene Expression (Butterworths, Boston, 1986) review selected topics in gene expression in E. coli, yeast, and mammalian cells; and Thilly, Mammalian Cell Technology (Butterworths, Boston, 1986) reviews mammalian expression systems.

[0030] Likewise, many reviews are available which describe techniques and conditions for linking and/or manipulating specific cDNAs and expression control sequences to create and/or modify expression vectors suitable for use with the present invention: e.g. Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, N.Y., 1982); Glover, DNA Cloning: A Practical Approach, Vol. I and II (IRL Press, Oxford, 1985), and Perbal, A Practical Guide to Molecular Cloning (John Wiley & Sons, N.Y., 1984).

[0031] Suitable expression systems for the invention include those disclosed by Itakura and Riggs, U.S. patent 4,704,362 (bacterial expression), by Clark et al., U.S. patent 4,675,285 and by Hamer U.S. patent 4,599,308 (mammalian expression), and by Kurjan et al., U.S. patent 4,546,082 (yeast expression).

[0032] Whenever SV40-based vectors are used, e.g. pcD vectors, a preferred host in the COS7 cell line, described by Gluzman, Cell, Vol. 23, pgs. 175-182 (1981) and available from the ATCC under accession number CRL1651.

[0033] Soluble FcγRIII of the invention are administered as a pharmaceutical composition for treating ITP. Such compositions contain an effective amount of the FcγRIII in a pharmaceutical carrier. A pharmaceutical carrier can be any compatible, non-toxic substance suitable for delivering the compositions of the invention to a patient. Generally, compositions useful for parenteral administration of such drugs are well known, e.g. Remington's Pharmaceutical Science, 15th Ed. (Mack Publishing Company, Easton, PA 1980). Alternatively, compositions of the invention may be introduced into a patient's body by an implantable drug delivery system, e.g. Urquhart et al., Ann. Rev. Pharmacol. Toxicol., Vol. 24, pgs. 199-236 (1984).

[0034] When administered parenterally, the soluble FcγRIII will be formulated in a unit dosage injectable

form (solution, suspension, emulsion) in association with a pharmaceutical carrier. Such carriers are inherently non-toxic and non-therapeutic. Examples of such carriers are normal saline, Ringer's solution, dextrose solution, and Hank's solution. Nonaqueous carriers such as fixed oils and ethyl oleate may also be used. A preferred carrier is 5% dextrose/saline. The carrier may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The soluble Fc γ RIII is preferably formulated in purified form substantially free of aggregates and other proteins at a concentration in the range of about 5 to 30 mg/ml, and preferably at a concentration in the range of about 10 to 20 mg/ml.

[0035] Selecting an administration regimen to deliver to a patient an amount of soluble Fc γ RIII which is effective in ameliorating the thrombopenia associated with ITP depends on several factors, including the serum turnover rate of the soluble Fc γ RIII, the serum level of competing endogenous Fc γ RIII associated with the immune disorder, the possible immunogenicity of the soluble Fc γ RIII, and the like. Preferably, an administration regimen maximizes the amount of soluble Fc γ RIII delivered to the patient consistent with an acceptable level of side effects. Accordingly, the amount of soluble Fc γ RIII delivered depends in part of the particular soluble Fc γ RIII employed and the severity of the disease being treated. Guidance in selecting appropriate doses is found in the literature on therapeutic uses of antibodies and antibody fragments, which are polypeptides of roughly the same size as the soluble Fc γ RIII, e.g. Bach et al., chapter 22, in Ferrone et al., eds., Handbook of Monoclonal Antibodies (Noges Publications, Park Ridge, NJ, 1985); and Russell, pgs. 303-357, and Smith et al., pgs. 365-389, in Haber et al., eds. Antibodies in Human Diagnosis and Therapy (Raven Press, New York, 1977). Preferably, the dose is in the range of about 1-20 mg/kg per day, more preferably about 1-10 mg/kg per day.

EXAMPLES

[0036] The following examples serve to illustrate the present invention. Selection of vectors and hosts as well as the concentration of reagents, temperatures, and the values of other variables are only to exemplify application of the present invention and are not to be considered limitations thereof.

Example I. Construction of stable mammalian cell transformants which express human Fc γ RIII

[0037] Preferably, mammalian cell lines capable of stable expression of the Fc γ RIII are produced by cotransfecting a host mammalian cell with a vector carrying a selectable marker and a vector carrying a host-compatible promoter and the Fc γ RIII cDNA insert. For pcD(SR α)-GP5, suitable hosts include Chinese ham-

ster ovary cells, COS monkey cells, and mouse L cells, such as a thymidine kinase deficient mutant (tk⁻) L cell available from the American Type Culture Collection under accession number CCL 1.3. The selectable marker allows one to select host cells which have a high probability of containing the Fc γ RIII gene fully integrated into the host genome. Typically, the ratio of pcD(SR α)-GP5 to the marker-containing vector in the transfection solution is about 10:1. Thus, if the marker gene is integrated into the host genome, it is very likely that pcD(SR α)-GP5 will also be integrated by virtue of its higher concentration. The selectable marker also provides a means of preventing the cultures of desirable transformants from being overgrown by revertant cells.

[0038] tk⁻ mouse L cells are cotransfected with pcD(SR α)-GP5 and pSV2tk, a pSV2 plasmid carrying a thymidine kinase gene under control of the SV40 early promoter. The pSV2 plasmid is described by Mulligan et al., Science, Vol. 209, pgs. 1422-1427 (1980), and by Subramani et al., Mol. Cell. Biol., Vol. 1, pgs. 854-864 (1981), and is available from the American Type Culture Collection under accession number 37146. Both plasmids are amplified in E. coli, e.g. strain HB101 available from the ATCC under accession number 33694, and purified by cesium chloride equilibrium centrifugation. A suspension of about 1 x 10⁵ of tk⁻ L cells in 10 ml of Dulbecco's Modified Eagle Medium (DME) with 10% fetal bovine serum is placed in a Falcon 3003 dish and cultured at 37°C for 20 hours in a 5% carbon dioxide gas incubator, after which the medium is replaced by 10 ml of fresh DME with 10% fetal bovine serum. The culture is incubated for an additional 4 hours. After incubation, 0.5 ml of solution A (50 mM Hepes, 280 mM NaCl, 1.5 mM sodium phosphate buffer, pH 7.22) and 0.5 ml of solution B (2M CaCl₂, 10 μ g pcD(SR α)-GP5, 1 μ g pSV2tk) are added to the culture medium, and the culture is incubated at 37°C for 24 hours in a 5% CO₂ atmosphere, after which the cells are placed in a selective medium with HAT (e.g. Sigma Chemical Co., St. Louis, MO). After two weeks the surviving colonies are subcloned by limiting dilution, and clones are assayed for expression of Fc γ RIII.

Example II. Use of stable L cell transformant expressing membrane-bound Fc γ RIII to measure serum levels of immune complex

[0039] A stably transformed mammalian cell expressing the Fc γ RIII of the invention can replace the Raji cell line in assays for immune complexes, e.g. Theofilopoulos et al., chapter 28, Manual of Clinical Laboratory Immunology, 3rd Ed. (American Society for Microbiology, Washington, D.C. 1986).

[0040] Antiserum to human IgG is prepared in rabbits, and the IgG fraction is isolated by ammonium sulfate precipitation followed by fractionation on an anion-exchange chromatography column (DEAE-cellulose 52; Whatman Chemical Separation Ltd., Maidstone, Eng-

land). Antiserum from commercial sources may also be used. The IgG fraction of the antiserum is brought to 5 mg/ml, and 1 ml is labeled with ^{125}I . After iodination and dialysis, the antiserum is diluted to 1 mg/ml with phosphate-buffered saline (PBS) to give a specific activity of about 3×10^5 cpm/ μg . Cells transformed with the Fc γ RIII cDNA are harvested after 72 hours of culture, and portions of 2×10^6 cells in 200 μl of medium are placed in 1.5-ml plastic Eppendorf[®] conical tubes (Brinkmann Instruments, Inc., Westbury, N.Y.; catalog no. 22-36-411-1). One ml. of Spinner medium (Eagle minimal medium without Ca^{2+} and Mg^{2+}) is added to each tube, and the cells are centrifuged at $800 \times g$ for 8 min. Supernatant fluids are aspirated, and the cell pellets are resuspended in 50 μl of Spinner medium. Serum to be tested is diluted fourfold in 0.15 M NaCl (physiological saline), and 25 μl is added to the transformed cells. After a 45-min incubation period at 37°C with gentle shaking by hand every 5 to 10 min, the cells are washed three times with Spinner medium. After the final wash, the cells, gently shaken every 5 to 10 minutes, are allowed to react for 30 min at 4°C with ^{125}I -labeled rabbit anti-human IgG diluted with Spinner medium containing 10 g of human serum albumin (HSA) per liter. After incubation, the cells are washed three times, supernatant fluids are completely aspirated, and radioactivity of the cell pellet is determined in a gamma counter. All assays are done in duplicate. The amount of uptake, expressed as absolute counts, percentage of the input, or micrograms of antibody, is referred to a standard curve of radioactive antibody uptake by cells incubated with normal human serum (complement source) containing various amounts of aggregated human IgG (AHG). The quantity of immune complex in serum is equated to an amount of AHG after correction for the dilution factor and is expressed as micrograms of AHG equivalent per milliliter of serum. The soluble AHG is formed from a solution of 6.5 mg of Cohn fraction II or isolated human IgG per ml of physiological saline, heated at 63°C for 30 min, and centrifuged ($1,500 \times g$, 15 min) to remove insoluble large aggregates. The supernatant is then diluted with buffer to yield a final concentration of approximately 1.6 mg/ml. Portions (0.5 ml) of this preparation are stored at -70°C and can be used for as long as 1 month.

[0041] The standard curve of radioactive antibody uptake is constructed as follows. Fifty-microliter portions of AHG (80 μg of protein) are serially diluted (11 twofold dilutions) in saline. Subsequently, 50 μl of a twofold dilution of normal human serum (source of complement), freshly obtained or stored at -70°C , is added to each dilution of AHG, mixed carefully, and incubated at 37°C for 30 min. Thereafter, 25 μl of each mixture is added to 2×10^6 cells in duplicate (fourfold final dilutions of serum containing from 20 μg to about 20 ng of AHG); the mixture is incubated, washed, and reacted with radiolabeled antibody. Radioactivity is then counted as with the test sera. A base line of radioactive antibody uptake

(background) by cells incubated with 25 μl of a fourfold dilution of normal human serum, used as a source of complement in the reference curve, is also established.

[0042] Standard preparations of IgG aggregates and of tetanus toxoid-human anti-tetanus toxoid immune complexes have recently been developed under the auspices of the International Union of Immunologists and are available through the Swiss Red Cross Blood Transfusion Services, e.g. Nydegger et al., *Clin. Exp. Immunol.*, Vol. 58, pgs. 502-509 (1984).

Example III. Construction of a soluble human Fc γ RIII

[0043] Soluble Fc γ RIII was constructed using site-specific oligonucleotide-directed mutagenesis of the Fc γ RIII cDNA insert of pcD(SR α)-GP5. The entire cDNA insert of pcD(SR α)-GP5 was subcloned as a 2.4 kilobase Bam HI fragment into the Bluescript KS plasmid (Stratagene, San Diego, CA) and single-stranded DNA was then prepared.

[0044] A synthetic oligonucleotide (minimum size about 18 nucleotides) encoding a TAA stop codon and a Bam HI site (with mutations shown in boldface in Figure 2) was used as a primer for complementary strand synthesis using DNA polymerase I large fragment (Amersham, Arlington Heights, IL). Figure 2 indicates the position of the stop codon and Bam HI site relative to the major domains encoded by the Fc γ RIII cDNA: S represents the region encoding the signal peptide, EC represents the region encoding the extracellular domain of Fc γ RIII, H represents the region encoding the hydrophobic, or transmembrane, domain of Fc γ RIII, and C represents the region encoding the cytoplasmic domain. Introducing the stop codon as indicated by Figure 2 produces a mutant Fc γ RIII lacking both the cytoplasmic and transmembrane domains; it is soluble as shown by the designation "sFc γ RIII" beside the modified DNA sequence. After the identity of the mutant was confirmed by DNA sequence analysis, it was subcloned back into the Bam HI site of the pcD(SR α) vector, amplified in *E. coli*, and transfected into COS 7 cells by electroporation. One day prior to transfection, approximately $(1.5-2.0) \times 10^6$ cos 7 monkey cells were seeded onto individual 100 mm plates in Dulbecco's modified Eagle's medium (DME) containing 6% fetal calf serum and 2 mM glutamine. To perform the transfection, cells were harvested by trypsinization and counted, washed twice in serum-free DME, and suspended to $(1-7) \times 10^6$ cells per ml in serum-free DME. DNA was added to 25 $\mu\text{g}/\text{ml}$ and the mixture was allowed to stand at room temperature for 10 minutes, after which 0.8 ml was pulsed in a 0.4 cm sterile cuvette with a Bio Rad[®] (Richmond, CA) Gene Pulser at 250 volts and 960 microfarads. After pulsing, cells were allowed to stand for 10 minutes and then were plated at $(1.5-2.0) \times 10^6$ per 100 mm plate in DME plus 6% fetal calf serum. Supernatants were harvested and assayed for soluble Fc γ RIII after 72 hours.

[0045] The soluble Fc γ RIII in the COS7 culture

supernatants was analyzed further by gel electrophoresis, and immunoprecipitated. A control, Experiment 1, and six actual experiments, Experiments 2 to 7, were performed. Experiments 1, 3 and 6 each contained proteins from culture supernatants of COS7 cells transfected with the pcD carrying the soluble FcγRIII cDNA. Proteins from Experiment 1 were immunoprecipitated with a non-specific mouse IgG₂ antibody; proteins from Experiment 3 were immunoprecipitated with human IgG, presumably by the binding of the Fc portion of the antibody with the soluble FcγRIII; and proteins from Experiment 6 were immunoprecipitated with the monoclonal antibody 3G8 which is specific for the extracellular domain of FcγRIII. The soluble FcγRIII corresponded to a broad band at about 40 kilodaltons. Experiments 4 and 7 contained proteins from culture supernatants of COS7 cells transfected with the pcD carrying the soluble FcγRIII cDNA and cultured in the presence of tunicamycin. Tunicamycin inhibits the post-translational attachment of N-linked carbohydrates to proteins. It was applied in an attempt to determine the nature of the apparent heterogeneity of the 40 kilodalton band. The tunicamycin caused the appearance of two bands of lower molecular weight, which is consistent with the deglycosylation of the soluble FcγRIII. Tunicamycin was added to the cultures as disclosed by Martens et al., PNAS 84: 809-813 (1987). Experiments 2 and 5 contained proteins from culture supernatants of COS7 cells which had undergone the same manipulations as the COS cells of Experiments 3 and 4 and Experiments 6 and 7, respectively, with the exception that no plasmid DNA was included in the transfection protocol.

Example IV Isolation of a variant human FcγRIII from Natural Killer Cells.

[0046] A cDNA library was constructed from mRNA extracted from a human natural killer (NK) cell line using the pcD(SRα) expression vector. The library was screened with a probe constructed from the cDNA insert of pcD(SRα)-GP5, which was radiolabeled with ³¹P by random-primed DNA labeling (Boehringer Mannheim, Indianapolis, IN). A clone pcD(SRα)-NL10 was obtained that exhibited human IgB binding activity. The sequence of the dDNA insert of pcD(SRα)-NL10 is illustrated in Figure 3. It can be seen that the amino acid sequence of this FcγRIII and that encoded by pcD(SRα)-GP5 are very similar, differing in the extracellular domain by only six amino acids.

[0047] The description of the foregoing embodiments of the invention have been presented for purpose of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise forms disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments are chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the

art to best utilize the invention in various embodiments and with such modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

[0048] Applicants have deposited pcD(SRα)-GP5 with the American Type Culture Collection Rockville, MD, USA (ATCC), under accession number 67707. This deposit is made under the Budapest Treaty for the deposit of Microorganisms; and under the conditions prescribed in the EPC and the implementing regulations thereto.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. An isolated nucleic acid having a sequence of nucleotides which encodes soluble or membrane-bound human FcγRIII.
2. An isolated nucleic acid according to Claim 1 wherein the nucleic acid is contained within pcD(SRα)-GP5, deposited under Accession number ATCC 67707.
3. A vector comprising a nucleic acid according to Claim 1 or Claim 2.
4. Use of a nucleic acid according to Claim 1 or Claim 2 in the production of an FcγRIII polypeptide.
5. A method of producing an FcγRIII polypeptide comprising the step of transforming a mammalian host cell line with a vector carrying a host-compatible promoter and a nucleic acid according to Claim 1 or Claim 2.
6. A host cell transformed with a vector according to Claim 3.
7. Recombinant production of FcγRIII polypeptide using a nucleic acid according to Claim 1 or Claim 2.

Claims for the following Contracting States : ES, GR

1. A method of producing an FcγRIII polypeptide comprising the steps of transforming a mammalian host cell line with a vector carrying a host-compatible promoter and a nucleic acid having a sequence of nucleotides which encodes soluble or membrane-bound human FcγRIII.
2. A method according to Claim 1 wherein the nucleic acid is contained within pcD(SRα)-GP5, deposited under Accession number ATCC 67707.

3. A method for the recombinant production of FcγRIII polypeptide comprising the step of using a nucleic acid having a sequence of nucleotides which encodes soluble or membrane-bound human FcγRIII.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Isolierte Nucleinsäure mit einer Sequenz von Nucleotiden, die einen löslichen oder membran- gebundenen human FcγRIII codiert.
2. Isolierte Nucleinsäure gemäß Anspruch 1, worin die Nucleinsäure in pcD(SRα)GP5 enthalten ist, hinterlegt unter der Zugangsnummer ATCC 67707.
3. Vektor, umfassend eine Nucleinsäure gemäß Anspruch 1 oder Anspruch 2.
4. Verwendung einer Nucleinsäure gemäß Anspruch 1 oder Anspruch 2 bei der Herstellung eines FcγRIII-Polypeptids.
5. Verfahren zur Herstellung eines FcγRIII-Polypeptids, umfassend den Schritt des Transformierens einer Säugerwirtszelle mit einem Vektor, der einen wirtskompatiblen Promoter und eine Nucleinsäure gemäß Anspruch 1 oder Anspruch 2 trägt.
6. Wirtszelle, die mit einem Vektor gemäß Anspruch 3 transformiert ist.
7. Rekombinante Produktion eines FcγRIII-Polypeptids unter Verwendung einer Nucleinsäure gemäß Anspruch 1 oder Anspruch 2.

Patentansprüche für folgende Vertragsstaaten : ES, GR

1. Verfahren zur Herstellung eines FcγRIII-Polypeptids, umfassend den Schritt des Transformierens einer Säugerwirtszelllinie mit einem Vektor, der einen wirtskompatiblen Promoter und eine Nucleinsäure mit einer Sequenz von Nucleotiden trägt, die löslich oder membrangebundenes human FcγRIII codiert.
2. Verfahren gemäß Anspruch 1, worin die Nucleinsäure in pcD(SRα)-GP5 enthalten ist, hinterlegt unter der Zugangsnummer ATCC 67707.
3. Verfahren zur rekombinanten Produktion eines FcγRIII-Polypeptids, umfassend den Schritt der Verwendung einer Nucleinsäure mit einer Sequenz von Nucleotiden, die lösliches oder membran-

gebundenes human FcγRIII codiert.

Revendications

5 Revendication pour les Etats contractants suivants : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Acide nucléique isolé ayant une séquence de nucléotides qui code FcγRIII humain soluble ou lié à la membrane.
2. Acide nucléique isolé selon la revendication 1 où l'acide nucléique est contenu dans pcD(SRα)-GP5 déposé sous le numéro de dépôt ATCC 67707.
3. Vecteur comprenant un acide nucléique selon la revendication 1 ou la revendication 2.
4. Utilisation d'un acide nucléique selon la revendication 1 ou la revendication 2 dans la production d'un polypeptide FcγRIII.
5. Procédé de production d'un polypeptide FcγRIII comprenant l'étape de transformation d'une lignée cellulaire hôte de mammifère avec un vecteur portant un promoteur compatible avec l'hôte et un acide nucléique selon la revendication 1 ou la revendication 2.
6. Cellule-hôte transformée avec un vecteur selon la revendication 3.
7. Production d'un polypeptide FcγRIII par recombinaison utilisant un acide nucléique selon la revendication 1 ou la revendication 2.

Revendications pour les Etats contractants suivants : ES, GR

1. Procédé de production d'un polypeptide FcγRIII comprenant les étapes de transformation d'une lignée cellulaire hôte de mammifère avec un vecteur portant un promoteur compatible avec l'hôte et un acide nucléique ayant une séquence de nucléotides qui code FcγRIII humain soluble ou lié à la membrane.
2. Procédé selon la revendication 1 où l'acide nucléique est contenu dans pcD(SRα)-GP5 déposé sous le numéro de dépôt ATCC 67707.
3. Procédé de production d'un polypeptide FcγRIII par recombinaison comprenant l'étape d'utilisation d'un acide nucléique ayant une séquence de nucléotides qui code FcγRIII humain soluble ou lié à la membrane.

10 30 50
 CACTCCAGTGTGGCATCATGTGGCAGCTGCTCCTCCCAACTGCTCTGCTACTT
 MetTrpGlnLeuLeuLeuProThrAlaLeuLeuLeu
 110 130 150
 GTTCCTGGAGCCTCAATGGTACAGGGTGCTCGAGAAGGACAGTGTGACTCTG
 lPheLeuGluProGlnTrpTyrArgValLeuGluLysAspSerValThrLeu
 210 230 250
 CACAATGAGAACCTCATCTCAAGCCAGGCCTCGAGCTACTTCATTGACGCTGCC
 HisAsnGluAsnLeuIleSerSerGlnAlaSerSerTyrPheIleAspAlaAla
 310 330 350
 CCCTCAGTGACCCGGTGCAGCTAGAAGTCCATGTCCGGCTGGCTGTTGCTCCAG
 hrLeuSerAspProValGlnLeuGluValHisValGlyTrpLeuLeuLeuGln
 410 430 450
 TCACAGCTGGAAGAACTGCTCTGCATAAGGTCACATATTTACAGAATGGC
 sHisSerTrpLysAsnThrAlaLeuHisLysValThrTyrLeuGlnAsnGly
 510 530 550
 GCCCACTCAAAGATAGCGGCTCCTACTTCTGCAGGGGGCTTGTGTTGGGAGTAAA
 AlaThrLeuLysAspSerGlySerTyrPheCysArgGlyLeuValGlySerLys
 610 630 650
 CAGTGTCAACCATCTCATCATTCTCTCCACCTGGGTACCAAGTCTCTTTCTGC
 laValSerThrIleSerSerPheSerProProGlyTyrGlnValSerPheCys
 710 730 750
 TGTGAAGACAAACATTTGAAGCTCAACAAGAGACTGGAAGGACCATAAACTTA
 rValLysThrAsnIleEnd

AATAAGAGCAGTGGCAGCAGCATCTCTGAACATTTCTCTGGATTTGCAACCCC
 GCCAGATCCTTTATCCAACCTCTCGATTTTTCCTTGGTCTCCAGTGGAAGGGAA
 CTAGAAATTGAAGTTTCAGAGCTACACAAACACTTTTTCTGTCCCAACCATTC
 CATACAAAAATTGCTCGTATTATAAATTACCCAGTTTAGAGGGGAAAAAAGAA
 AGGACCCTACAGAGTGTGGGAACTGCTGGGGATCTAGAGAATTCAGTGGGACC
 GGGAGGTGTTCCCATCTGAGCCCAGAGATAAGGGTGTCTTCCTAGAACATTAG
 CTTCCAGGGGACTCTATCAGAACTGGACCATTTCCAAGTATATAACGATGAGC
 TTGGGGTGGGGGTGGGGTGGAAAAGAAAGTACAGAACAAACCCTGTGTCACTG
 AATGAGAAAGCCTGAGAAGAAAGAACCAACCACAAGCACACAGGAAGGAAAGC
 TTAATGCAGGGACTGTAAAACCACTTTTCTGCTTCAATGTCTAGTTCCTGTA
 TAAATGTACTACTGAGCTTCACTGAGTTACGCTGTGAAACTTTCAAATCCTTC
 ATCAAAAAAGCTTTAGCTGTCTCCTGTTTTGTAAGCTTTCAGTGCAACATTT

FIGURE 1 PART A

70 +1 90
 CTAGTTTCAGCTGGCATGCGGACTGAAGATCTCCCAAAGGCTGTGGT
 LeuValSerAlaGlyMetArgThrGluAspLeuProLysAlaValVa
 170 190
 AAGTGCCAGGGAGCCTACTCCCCTGAGGACAATTCCACACAGTGGTTT
 LysCysGlnGlyAlaTyrSerProGluAspAsnSerThrGlnTrpPhe
 270 290
 ACAGTCGACGACAGTGGAGAGTACAGGTGCCAGACAAACCTCTCCA
 ThrValAspAspSerGlyGluTyrArgCysGlnThrAsnLeuSerT
 370 390
 GCCCCTCGGTGGGTGTTCAAGGAGGAAGACCCTATTCACCTGAGGTG
 AlaProArgTrpValPheLysGluGluAspProIleHisLeuArgCy
 470 490
 AAAGACAGGAAGTATTTTCATCATAATTCTGACTTCCACATTCCAAAA
 LysAspArgLysTyrPheHisHisAsnSerAspPheHisIleProLys
 570 590
 AATGTGTCTTCAGAGACTGTGAACATCACCATCACTCAAGGTTTGG
 AsnValSerSerGluThrValAsnIleThrIleThrGlnGlyLeuA
 670 690
 TTGGTGATGGTACTCCTTTTTGCAGTGGACACAGGACTATATTTCTC
 LeuValMetValLeuLeuPheAlaValAspThrGlyLeuTyrPheSe
 770 790
 AATGGAGAAAGGACCCTCAAGACAAATGACCCCCATCCCATGGGAGT

 ATCATCCTCAGGCCTCTCTACAAGCAGCAGGAAACATAGAAGTCTAGA 1000
 AAGCCCATGATCTTCAAGCAGGGAAGCCCCAGTGAGTAGCTGCATTG
 CCTCACAGTAAAAACAATAACAGGCTAGGGATGGTAATCCTTTAAA
 AATAATTATTCCTAAACAAATGGATAAGTAGAATTAATGATTGAGGC 1200
 AATGAAAGCATGGCTGAGAAATAGCAGGGTAGTCCAGGATAGTTTAA
 CCGTAGTGGAATTAACAGGAAATCATGAGGGTGACGTAGAATTGAGT 1400
 CCTCTAATGCTAGGAGTAGCAAATGGTCCTAGGAAGGGGACTGAGGA
 TCCCAAGTTAAGCTAAGTGAACAGAACTATCTCAGCATCAGAATGAG 1600
 GCAGGAGGTGAAAATGCTTTCTTGGCCAGGGTAGTAAGAATTAGAGG
 TAGCTTTGTTTCATTGCATTTATTAAACAAATGTTGTATAACCAATAC 1800
 TTCAGTCAGTTCCAATGAGGTGGGGATGGAGAAGACAATTGTTGCTT
 CTTGGTTCCAATAAAGCATTTTACA (A)₂

FIGURE 1 PART B

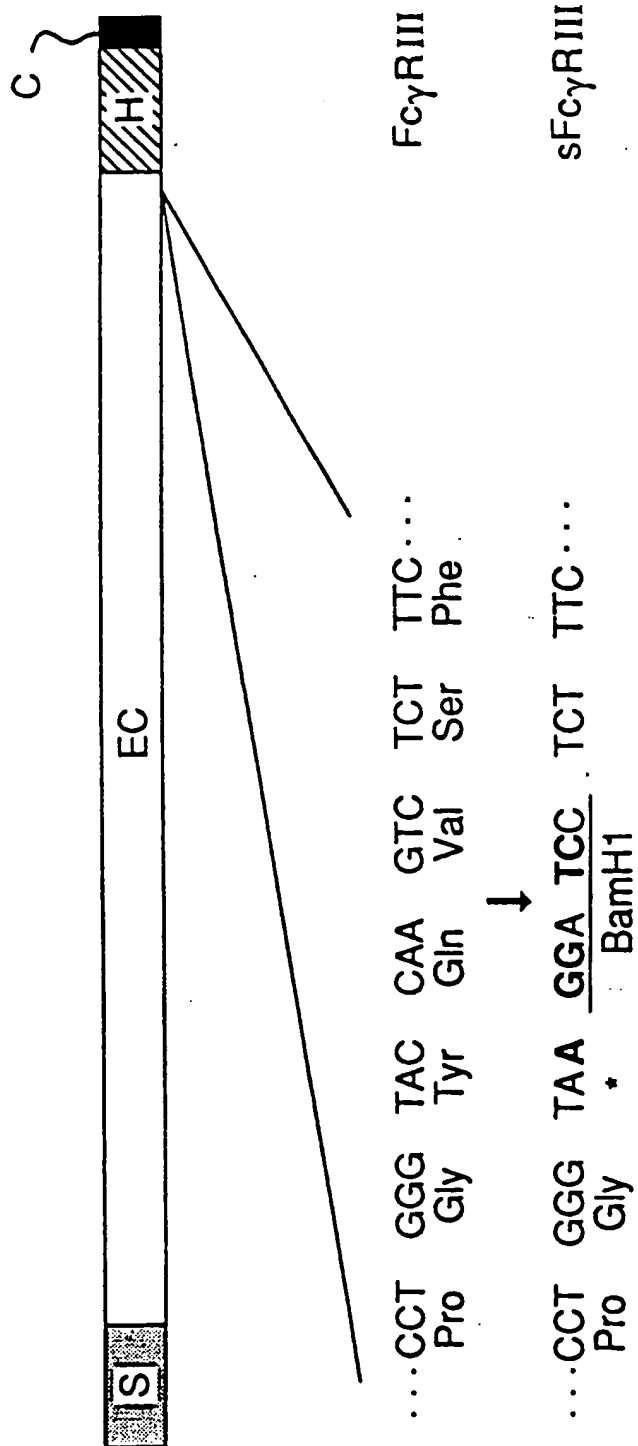


FIGURE 2

NL 10

```

      10      30      50
GGCGGGGGGGGGGGTAAATCCGCAGGACCTGGGTAACACGAGGAAGGGCTCCG

      110      130      150
TGCTCCTCCCAACTGCTCTGCTACTTCTAGTTTCAGCTGGCATGCGGACTGAA
euLeuLeuProThrAlaLeuLeuLeuLeuValSerAlaGlyMetArgThrGlu
      210      230      250
GCTCGAGAAGGACACTGTGACTCTGAAGTGCCAGGGAGCCTACTCCCCTGAG
lLeuGluLysAspSerValThrLeuLysCysGlnGlyAlaTyrSerProGlu
      310      330      350
GCCTCGAGCTACTTCATTGACGCTGCCACAGTCGACGACAGTGGAGAGTACAGG
AlaSerSerTyrPheIleAspAlaAlaThrValAspAspSerGlyGluTyrArg
      410      430      450
TCCATATCGGCTGGCTGTTGCTCCAGGCCCTCGGTGGGTGTTCAAGGAGGAA
alHisIleGlyTrpLeuLeuLeuGlnAlaProArgTrpValPheLysGluGlu
      510      530      550
TAAGGTACACATATTTACAGAATGGCAAAGGCAGGAAGTATTTTCATCATAAT
sLysValThrTyrLeuGlnAsnGlyLysGlyArgLysTyrPheHisHisAsn
      610      630      650
TTCTGCAGGGGGCTTTTTGGGAGTAAAAATGTGTCTTCAGAGACTGTGAACATC
PheCysArgGlyLeuPheGlySerLysAsnValSerSerGluThrValAsnIle
      710      730      750
CACCTGGGTACCAAGTCTCTTTCTGCTTGGTGATGGTACTCCTTTTTGCACTG
roProGlyTyrGlnValSerPheCysLeuValMetValLeuLeuPheAlaVal
      810      830      850
AAGAGACTGGAAGGACCATAAAATTTAAATCGAGAAAGGACCCTCAAGACAAATGA
rArgAspTrpLysAspHisLysPheLysTrpArgLysAspProGlnAspLysEnd

GAACATTTCTCTGGATTTTGCAACCCCATCATCCTCAGGCCTCTCTACAAGCAGCA
TTTCCTTTGCTCTCCAGTGGAAGGGAAAAGCCCATGATCTTCAAGCAGGGAAGCCC
AAACACTTTTTCTGTCCCAACCGTTCCCTCACAGCAAAGCAACAATACAGGCTAG
TACCCAGTTTAGAGGGGAAAAAAAAACAATTATTCCTAAATAAATGGATAAGTAG
GGGATCTAGGGAATTCAGTGGGACCAATGAAAGCATGGCTGAGAAATAGCAGGTA
AAGGGTGTCTTCCTAGAACATTAGCCGTAGTGGAATTAACAGGAAATCATGAGGG
ATCTCCAAGTATATAACGATGAGTCCTCTTAATGCTAGGAGTAGAAAATGGTCCT
AGTACAGAACAAAACCTGTGTCACTGTCCCAAGTTGCTAAGTGAACAGAACTATC
GCACACAGGAAGGGGGGGCGCAGGAGGTGAAAATGCTTTCTTGGCCAGGGTAGTAA
CAATATCTAATTCCTGTGTAGCTTTGTTTCATTGCATTTATTAACAAATGTTGTA
GAACTTTCAAATCCTTCATCATGTCACTTCCAATGAGGTGGGGATGGAGAAGAC
GCTTTAAGCGCAACATTTCTTGGTTCCAATAAAGCATTTTACAAGATCTTGCATG
ATGATAAAAAAAAAAAAAAAAAAAAAA

```

FIGURE 3 PART A

NL 10

70 90
 GATATCTTTGGTGACTTGTCCACTCCAGTGTGGCATCATGTGGCAGC
 MetTrpGlnL
 170 190
 GATCTCCCAAAGGCTGTGGTGTTCCTGGAGCCTCAATGGTACAGGGT
 AspLeuProLysAlaValValPheLeuGluProGlnTrpTyrArgVa
 270 290
 GACAATTCCACACAGTGGTTTCACAATGAGAGCCTCATCTCAAGCCAG
 AspAsnSerThrGlnTrpPheHisAsnGluSerLeuIleSerSerGln
 370 390
 TGCCAGACAAACCTCTCCACCCTCAGTGACCCGGTGCAGCTAGAAG
 CysGlnThrAsnLeuSerThrLeuSerAspProValGlnLeuGluV
 470 490
 GACCCTATTACCTGAGGTGTACAGCTGGAAGAACACTGCTCTGCA
 AspProIleHisLeuArgCysHisSerTrpLysAsnThrAlaLeuHi
 570 590
 TCTGAOCTTCTACATTCCAAAAGCCACACTCAAAGACAGCGGCTCCTAC
 SerAspPheTyrIleProLysAlaThrLeuLysAspSerGlySerTyr
 670 690
 ACCATCACTCAAGGTTTGGCAGTGTCAACCATCTCATCATTCTTTC
 ThrIleThrGlnGlyLeuAlaValSerThrIleSerSerPhePheP
 770 790
 GACACAGGACTATATTTCTGTGAAGACAAACATTGGAAGCTCAAC
 AspThrGlyLeuTyrPheSerValLysThrAsnIleArgSerSerTh
 870 890
 CCCCCATCCCATGGGGTAATAAGAGCAGTAGCAGCAGCATCTCT

 GGAAACATAGAAGCTCAGAGCCAGATCCCTTATCCAACTCTCGACT 1000
 CAGTGAGTAGCTGCATTCCCTAGAAAATTGAAGTTTCAGAGCTACAC
 GGATGGTAATCCTTTAAACATACAAAAATTGCTCGTCTTATAAAT 1200
 AATTAATGGTTGAGGCCAGGACCATACAGAGTGTGGGAAGTGTG
 GTCCAGGATAGTCTAAGGGAGGTGTTCCCATCTGAGCCCAGAGAT 1400
 TGACGTAGAATTGAGTCTTCCAGGGGACTCTATCAGAAGTGGACC
 AGGAAGGGGACTGAGGATTGCGGTGGGGGCTGGGGTGGAAAAGAA 1600
 TCAGCATCAGAATGAGAAAGCCTGAGAAGAAAGAACCAACCACAA
 GAATTAGAGGTTAATGCAGGGACTGTAAAACCACTTTTCTGCTT 1800
 TAACCAATACTAAATGTACTACTGAGCTTCGCTGAGTTAAGTTAT
 AATTGTTGCTTATGAAAGAAAGCTTTACGTGTCTCTCTTTTGTAA 2000
 CTA CTCTTAGATAGAAGATGGGAAAACCATGGTAATAAAATATGA

FIGURE 3 PART B

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.